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Journal

Toxicology in vitro : an international journal published in association with BIBRA, 28(5)

ISSN

0887-2333

Authors

Cervellati, F
Muresan, XM
Sticozzi, C
et al.

Publication Date

2014-08-01

DOI

10.1016/j.tiv.2014.04.012

Peer reviewed

Published in final edited form as:

Toxicol In Vitro. 2014 August ; 28(5): 999–1005. doi:10.1016/j.tiv.2014.04.012.

Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells

Franco Cervellati¹, Ximena Muresan¹, Claudia Sticozzi¹, Roberto Gambari¹, Giulia Montagner¹, Henry Forman^{2,3}, Claudia Torricelli⁴, Emanuela Maioli⁴, and Giuseppe Valacchi^{1,2,3,4,5,*}

¹Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy.

²Davis School of Gerontology, University of Southern California - USA

³Life and Environmental Sciences Unit, University of California at Merced - USA

⁴ Department of Life Sciences, University of Siena, Siena, Italy.

⁵ Kyung Hee University, Department of Food and Nutrition, Seoul, South Korea

Abstract

Background—Information about the harmful effects of vaping is sparse and inconsistent, therefore, since the use of electronic cigarettes (e-CIGs) has become increasingly popular as a tool to limit tobacco smoking, it is urgent to establish the safety or the toxicity of the liquid vaporized by the atomizer of the commercial e-CIGs.

Methods—Skin (HaCaT) and lung (A549) cells, the main targets of cigarette smoke, were exposed to e-CIG vapor (e-CIG Mini Touch T-Fumo T-TEX) and cigarette smoke (UK research cigarette) in a smoke chamber in vitro. The cytotoxic effect of the exposure was analyzed in both cell types by ultrastructural morphology, Trypan Blue exclusion test and LDH assay. In addition, pro-inflammatory cytokines were measured in culture medium by the Bio-Plex cytokine assay kit.

Results—The cytotoxic components of e-CIG were restrained to the flavoring compound and, to a lesser extent, to nicotine and their effects were comparable to that of cigarette smoke. Humectants alone exhibited no cytotoxicity but induced the release of cytokines and pro-inflammatory mediators, mainly in keratinocytes.

Conclusions—Based on our results, we can state that e-CIG vapors exposure is not completely harmless, although far less toxic than CS. In fact, besides the deleterious effect of flavor and nicotine, even the humectants alone are able to evoke some adverse cellular events, such as enhanced cytokines release. This study will hopefully promote the development of truly innocuous e-CIGs to help people quit smoking.

Keywords

e-CIG vapor; tobacco cigarette smoke; skin and lung cells; cytotoxicity; cytokines release

*Corresponding Author: Giuseppe Valacchi, PhD Department of Life Sciences and Biotechnology University of Ferrara, Via Borsari, 46 – 44121 - Ferrara, Italy Giuseppe.valacchi@unife.it Phone: + 39 0532 455482.

INTRODUCTION

Developed in China in 2004, the electronic cigarette (e-CIG) has become increasingly popular in numerous other countries. Marketers of the e-CIG describe it as an aid to help people quit smoking. They claim that while using an e-CIG simulates tobacco cigarette smoking, the odor and risks associated with tobacco smoke are eliminated as no combustion products and no tobacco toxins are inhaled [1].

In fact, in addition to variable doses of nicotine and different flavors, the base liquid typically includes propylene glycol (PG) and/or glycerol (also called vegetable glycerin or VG) and/or polyethylene glycol 400 (PEG400), all of which are widely used as additives in foods and personal care products, such as toothpaste [2].

Thus, the components of e-CIG vapors, inhaled in the act now called vaping, are assumed to be less harmful than the thousands of known and unknown toxicants in tobacco smoke. Nonetheless, this assumption does not entirely rule out potentially deleterious effects of inhaling the vapor of the nicotine/flavor mixture. Indeed, while The World Health Organization (WHO) has not excluded the e-CIG might be useful as a smoking cessation aid, it has stated that current research does not warrant the conclusion that the e-CIG is as safe and effective, in reducing nicotine-related withdrawal symptoms, as nicotine-replacement patches or gum [3]. There is no specific legislation on the use of e-CIGs in Europe and currently member countries set their own regulations. Some countries, such as Belgium and Denmark, banned the sale of e-CIGs while Germany and Austria classified the e-CIG as a medical product. In the Netherlands, the use and purchase of e-CIGs is legal, but advertisement of them is banned. The European Union however, is currently debating banning all smokeless tobacco throughout Europe. Taking these products off the market however, would force thousands of users, who positively experienced vaping,[4] to return to cigarette smoking, with the known deleterious effects. Therefore, it is urgent to establish the safety or the toxicity of the components of the vapors from commercial e-CIGs in order to provide legislators, manufacturers and smokers with the essential scientific information required to make informed decisions. In the current study, we compared the in vitro cytotoxicity of cigarette smoke and e-CIG vapors on cells from lung and skin, the organs directly targeted by tobacco cigarettes.[5, 6]

Short term exposure of HaCaT cells (keratinocytes) and A549 cells (lung epithelial cells) to tobacco smoke and e-CIG vapors with and without aroma or nicotine were carried out. The results revealed that e-CIG vapors have some toxic effect on cell viability. In particular, the harmful component of the e-CIG seems to be restrained to the flavoring compounds rather than to nicotine and humectants. In addition, screening of an array of cytokines released from the cells exposed to e-CIG vapors without additives showed that the basal components alone are able to induce the release of several cytokines and pro-inflammatory mediators, suggesting the even humectants might have a potential, although non-cytotoxic, harmful effect.

METHODS

Cell culture

HaCaT cells, (a gift from Dr. F. Virgili), were grown in Dulbecco's modified Eagle's medium High Glucose (Lonza, Milan, Italy), supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. A549 cells were purchased from ATCC (Rockville, MD). Ham's F-12, foetal bovine serum, RPMI-1640, penicillin/streptomycin and L-glutamine were obtained from Lonza (Milan, Italy). Cell suspension containing 5×10^6 viable cells/ml were used. Cells were incubated at 37 °C for 24 h in 95% air/5% CO₂ until 80% confluency.

Cell viability

Viability studies were performed at different times after exposure by Trypan Blue exclusion test and LDH release assay. After Trypan blue staining, cells were counted by a cell counter (Invitrogen, Monza, Italy). Viable and nonviable cells were recorded separately, and the means of three independent counts were pooled for analysis and expressed as percent of died cells with respect to total cell number. The LDH release was measured by a two steps enzymatic assay. In the first step, NAD⁺ is reduced to NADH/H⁺ by the LDH-catalyzed conversion of lactate to pyruvate; in the second step, the catalyst (diaphorase) transfers H/H⁺ from NADH/H⁺ to tetrazolium salt which is reduced to formazan. The amounts of LDH in the culture medium were determined and calculated according to kit instructions (EuroClone Milan, Italy). Prior to each assay, the cells were lysed with 2% (V/V) Triton X-100 in culture media for 30 min at 37°C to obtain a representative maximal LDH release as the positive control with 100% toxicity. All tests were performed in triplicate and assay was repeated three times independently with similar results.

CS and e-CIG exposure

Prior to CS and e-CIG exposure of the cells, medium was aspirated and fresh serum-free medium was added. Cells were then exposed for 50 min to CS and e-CIG mixture. Control cells were exposed to filtered air for the same duration (50 min) after changing media. The time and the method of exposure were chosen based on our previous works.[5,7]

HaCaT cells were exposed to fresh CS in an exposure system that generated CS by burning one UK research cigarette (12 mg tar, 1.1 mg nicotine), and to e-CIG mixtures (balsamic flavours with or without nicotine, Cloudsmoke, Terna Trade) using a vacuum pump to draw air through the cigarette and leading the smoke stream over the cell cultures as described previously by our group.[5,7] After the exposure (air or CS, e-CIG), fresh media supplemented with 10% FBS was added to the cells.

Ultrastructural study

After CS exposure, HaCaT and A549 cells (1×10^6 cell/ml) were scraped and collected in 0.1M cacodylate buffer (pH 7.4), then spun in 1.5ml tubes at 2000×g for 5 min. Pellets were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 4 h at 4 °C. They were then washed with 0.1M cacodylate buffer (pH 7.4) three times and post-fixed in 1% osmium tetroxide and 0.1M cacodylate buffer at pH 7.4 for 1 h at room temperature. The specimens

were dehydrated in graded concentrations of ethanol and embedded in epoxide resin (Agar Scientific, 66A Cambridge Road, Stanstead Essex, CM24 8DA, UK). Cells were then transferred to latex modules filled with resin and subsequently thermally cured at 60 °C for 48 h. Semi-thin sections (0.5-1 µm thickness) were cut using an ultra-microtome (Reichert Ultracut S, Austria) stained with toluidine blue, and blocks were selected for thinning. Ultra-thin sections of about 40-60 nm were cut and mounted onto formvar-coated copper grids. These were then double-stained with 1% uranyl acetate and 0.1% lead citrate for 30 min each and examined under a transmission electron microscope, Hitachi H-800 (Tokyo, Japan), at an accelerating voltage of 100 kV.

Cytokine Assay

Cytokines released in culture medium by HACAT and A549 cells were measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA) as described elsewhere.[8, 9]

The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 µl of sample. In our experiments, we used the premixed multiplex beads of the Bio-Plex human cytokine Human 27-Plex Panel (Bio-Rad, Cat. n.o. 171-A11127), which included twenty-seven cytokines [IL-1b; IL-1ras, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-10, IL-12 (P70), IL-13, IL-15, IL-17, Basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, IP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF]. Briefly, 50 µl of cytokine standards or samples (supernatants from treated cells) were incubated with 50 µl of anti-cytokine conjugated beads in 96-well filter plates for 30 min at room temperature with shaking. Plates were then washed by vacuum filtration three times with 100 µl of Bio-Plex wash buffer, 25 µl of diluted detection antibody was added, and plates were incubated for 30 min at room temperature with shaking. After three filter washes, 50 µl of streptavidinphycoerythrin was added, and the plates were incubated for 10 min at room temperature with shaking. Finally, plates were washed by vacuum filtration three times, beads were suspended in Bio-Plex assay buffer, and sample were analyzed on Bio-Rad 96 plate reader using the Bio-Plex suspension array system and Bio-Plex manager software (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Values are expressed as the mean±SD values from at least three independent experiments. Student's t was used to determine statistical significance with a threshold of p values less than 0.05.

RESULTS

LDH release and viability

As shown in Fig.1A and Fig.2A respectively, HaCaT and A549 cells viability does not change in controls over 24 h and, consistently, a steady low release of LDH was observed (Fig.3A and Fig.4A). In contrast, exposure to CS caused an early (6 h) and progressive decrease in cell viability (Fig.1B and Fig.2B) and increased LDH release (Fig.3B and Fig. 4B) with a similar trend during the different time points in both cell lines, although

keratinocytes seem to be more susceptible to CS induced toxicity after 24hr (Fig.4B). Exposure to e-CIG vapor in which both flavoring substances and nicotine were absent resulted in no change in either cell viability (Fig 1C and Fig 2C) or LDH release (Fig.3C and Fig.4C) over 24 h. In stark contrast to e-CIG without the additives, exposure to e-CIG with flavor caused significant progressive loss of viability (Fig.1D and Fig.2D) and increased LDH release (Fig.3D and Fig.4D) in both cell types. Even more dramatically, e-CIG with flavoring and nicotine caused rapid (50 min) and marked loss in viability (Fig.1E and Fig. 2E) and enhanced LDH release (Fig.3E and Fig.4E), exhibiting a quantitative and qualitative response superimposable to that of CS exposure.

Cellular morphology

Results from TEM are in line with the viability and toxicity data. As shown in Fig.5, in control conditions, the ultrastructural appearance is unchanged after both 50 minutes (T0) and 24 hours (T24); cells appear closely adherent and cellular organelles and mitochondria are well defined in both cell types. The morphology of the cells exposed to CS shows clear signs of cellular damage and presence of vacuoles. Once the cells were exposed to e-CIG with flavors it is possible to observe an increase in vacuolization and alteration of cytoplasmic membrane. The degeneration of intracellular organelles is worsened in the presence of flavors plus nicotine and particularly evident in HaCaT cells, with a marked vacuolization consequent to the expansion of the mitochondria and the endoplasmic reticulum. The cells treated with e-CIG without nicotine and flavors (humectants alone), remained intact with the same ultrastructural aspect of control cells, even 24 hours after treatment.

Cytokine release

Despite the lack of cytotoxicity of the basal component of e-CIG vapors, a possible irritant/inflammatory effect could not be excluded. Therefore, we performed a quantitative measurement using the Bio-plex technology [8, 9] of multiple cytokines released by both keratinocytes and lung cells after vapors exposure. The results concerning the released cytokines/chemokines displaying concentrations > 5 pg/ml in both HaCaT and A549 cells were taken in consideration in order to minimize the background, and are reported in Table 1. As expected, the pattern of cytokine/chemokine release is different in the two cell lines. Interestingly, increases of cytokine/chemokine release (> 1.2 fold) was found in the two cell lines when PDGF-BB, basic FGF, IL-8, IL-12, IL-17, GM-CSF, IP-10, MCP-1 and MIP-1 β were analyzed. Increased release of IL-1 α , IL-10, G-CSF, IFN- γ , RANTES, TNF- α and VEGF was found only in HaCaT. IL-6 release decreased both in HaCaT and A549 cells. The highest increase in HaCaT cells (> 8 fold) was that of IL-8 (11.9 fold) and IP-10 (13.7 fold). The highest increase in A549 cells was that of basic FGF (9.9 fold). These results indicate that the basal components of e-CIG vapor, although non-toxic, contain some pro-inflammatory stimuli leading to a changes in the secretome pattern depending from the employed cells lines. We also found fluctuations in cytokines release after other e-CIG and CS exposures but, because of the concomitant cell death, interpretation of such changes as active release or loss due to cell disruption was not possible (data not shown).

DISCUSSION

Although there have been few studies suggestive of harmful effects from vaping, the results thus far have been inconsistent. This is likely due to a lack of standardized assessment, but also to varying chemical composition of commercial refill fluids among brands. A recent study showed that using an e-CIG for 5 min has immediate adverse physiologic effects similar to those seen with tobacco smoking.[10] In another recent study, where serum cotinine, lung function, exhaled carbon monoxide and nitric oxide were assessed and compared between e-CIG users and tobacco cigarette smokers, the results showed that e-CIGs generated smaller changes in lung function but had a nicotinic impact similar to that from tobacco cigarettes.[11] Although it is generally believed that the diseases caused by smoking are more likely caused by products of combustion rather than by nicotine, nicotine inhalation through e-CIGs can be completely avoided by simply using nicotine-free cartridges. Our results are in agreement with the study by Bahl et al. performed in vitro on embryonic and adult cells who found that cytotoxicity was not due to nicotine or humectants, but was correlated with the number and concentration of chemicals used to flavor fluids[12]. Nevertheless, the absence of cytotoxicity of humectants on skin and lung cells does not exclude possible harmful effects on other cell populations, such as pulmonary macrophages, especially after long term exposure. Indeed, an isolated case of lipoid pneumonia associated with vaping and ascribed to the glycerin vapors has been reported in a 42-years-old woman who had used e-CIGs for about 7 months [13]. On the other hand, glycerin and propylene glycol are commonly used as humectants also in tobacco cigarettes, to prevent excessive drying of the tobacco filler and in water pipe tobacco, to increase smoke development. In a previous in vivo study, performed in a rat model, the addition of glycerin and propylene glycol to tobacco cigarettes was found do not significantly affect the toxicity of smoke inhaled for 13 weeks.[14] However, despite the likely safety of humectants, the presence of silicate particles and metal elements, which have been recently found in vapours from an e-CIG leading brand, may have some cytotoxic effects.[15] The authors of this last study recommend the manufacturers high selection of the materials used in e-CIGs and stringent quality control procedures, since the metals are thought to derive from the cartomizer (atomizer and cartridge), rather than from the fluid. A comparative study on the effects of e-CIG vapor and cigarette smoke on indoor air quality,[16] provided evidence that vaping is safer than smoking and that “secondhand” vapor is much less dangerous than secondhand smoke.[17] Cahn and Siegel provided a table of the components of several brands of e-CIGs.[18] While some of the components other than flavoring agents and nicotine are generally regarded as safe, including propylene glycol and glycerin, information on some components does not clearly rule out potential toxicity. Although the present results and the bulk of the literature indicate that e-CIG without additives are likely devoid of cytotoxicity, such results probably underestimated their whole harmful potential since toxicity tests do not routinely evaluate possible inflammatory and irritant effects of vaping. The current study showed, for the first time, that a panel of pro-inflammatory cytokines/chemokines (PDGF-BB, basic FGF, IL-8, IL-12, IL-17, GM-CSF, IP-10, MCP-1 and MIP-1 β) are increasingly released into the medium, following exposure to e-CIG vapors without additives, by keratinocytes and lung cells, indicating that even the non-cytotoxic components of e-CIG might have a potential dangerous effect. The release of other

cytokines, such as IL-1 α , IL-10, G-CSF, IFN- γ , RANTES, TNF- α and VEGF instead increased only in the keratinocyte cell line. We don't know whether all e-CIGs contain inflammatory stimuli, but it would be prudent if such studies were carried out while the use of e-CIGs is becoming more common so that brands can be developed that are truly innocuous. Moreover, the experimental system employed might be useful to identify molecules able to decrease the pro-inflammatory effects of eCIG. Interestingly, for each brand a differential chemical strategy to block pro-inflammatory effects might be proposed.

What this paper adds

In closing, our results indicate that e-CIGs are illusively safety, although they are far less noxious than tobacco cigarettes, thus the ideal remains that people should stop both smoking and vaping. In fact even the only vaping is able to stimulate cytokines release that play a role in inflammation. Nevertheless, this study also suggests that, if the refill accessories are properly chosen, vaping could be a safer alternative to smoking for smokers who are unable or unwilling to stop.

Acknowledgments

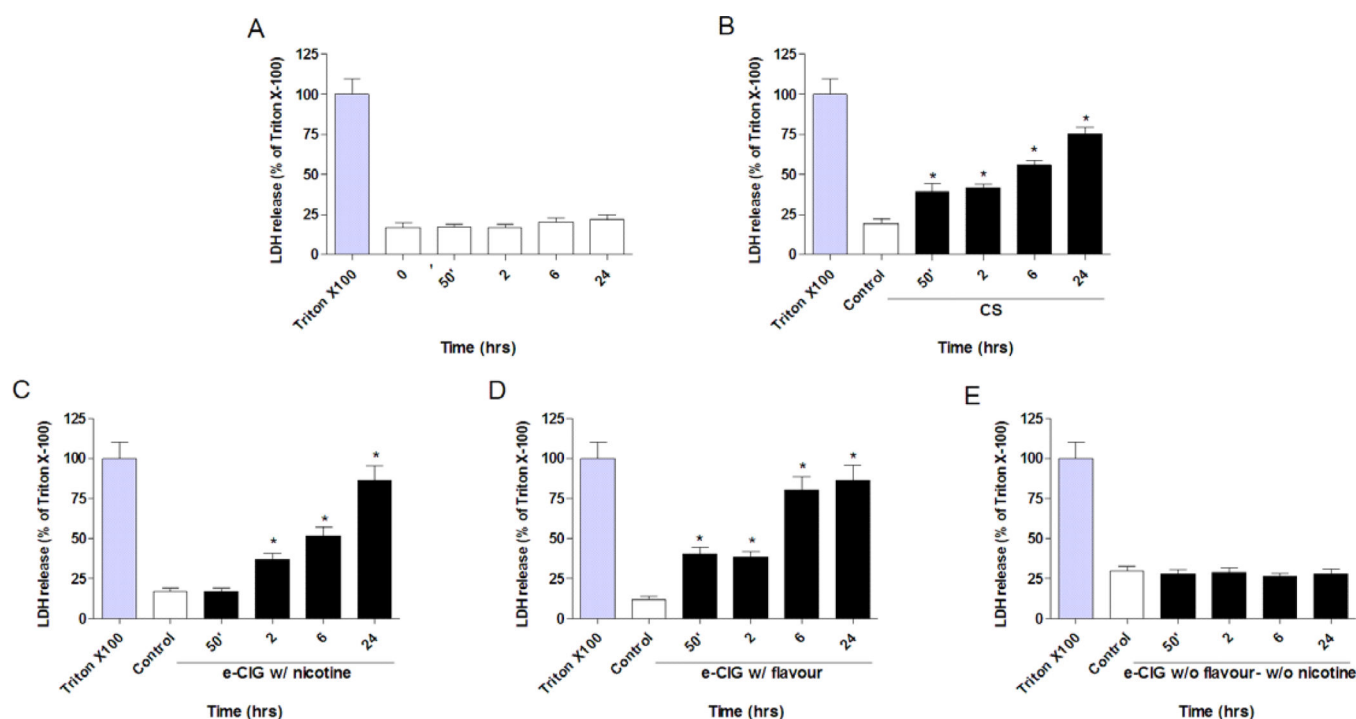
The authors thank Marangoni's Pharmacy, Argenta, Italy for the kind gift of e-CIG Mini Touch T-Fumo T-TEX srl.

Funding HJF is supported by NIH grant ES020942.

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**Figure 1.**

Cytotoxicity measured as LDH release in HaCaT cells after exposure to air (A), cigarette smoke (B), electronic cigarette with nicotine (C), electronic cigarette with flavor (D) and only vaping (E). Triton X represent 100% of LDH release. Data are expressed as percentage of control (averages of five different experiments), * $p < 0.05$).

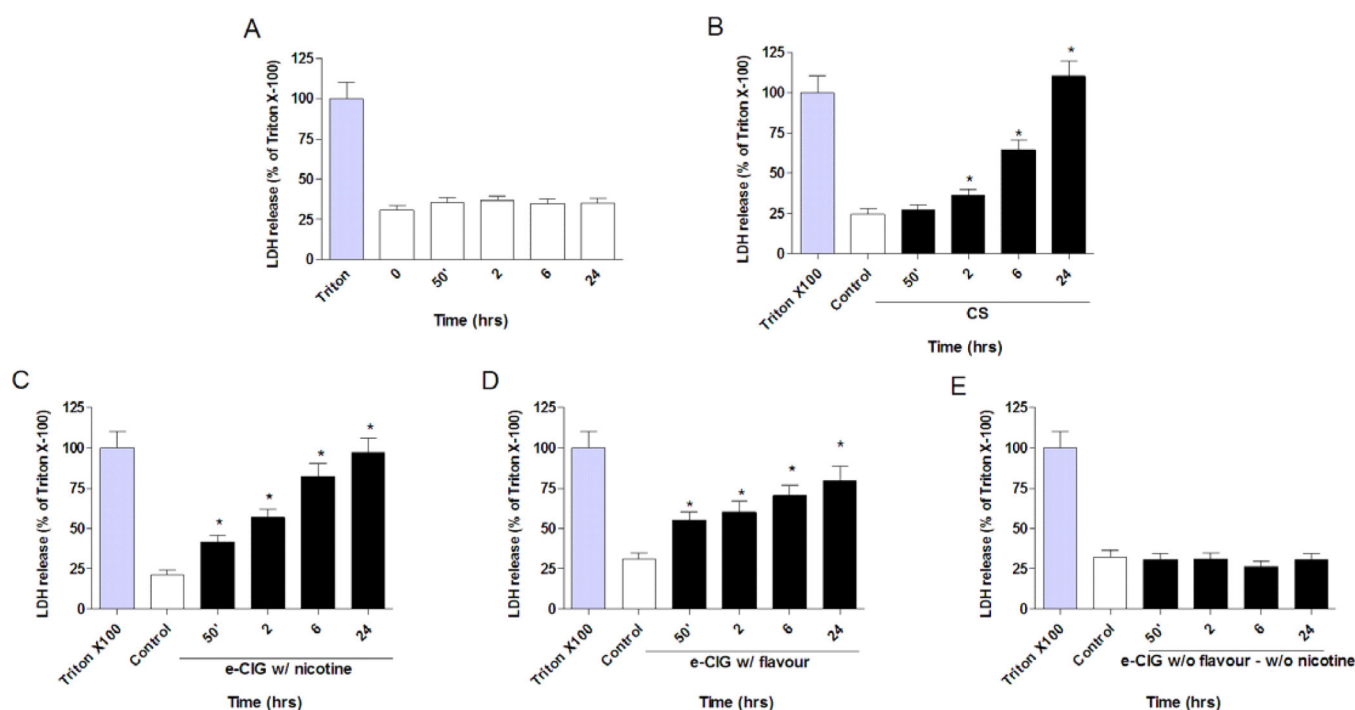
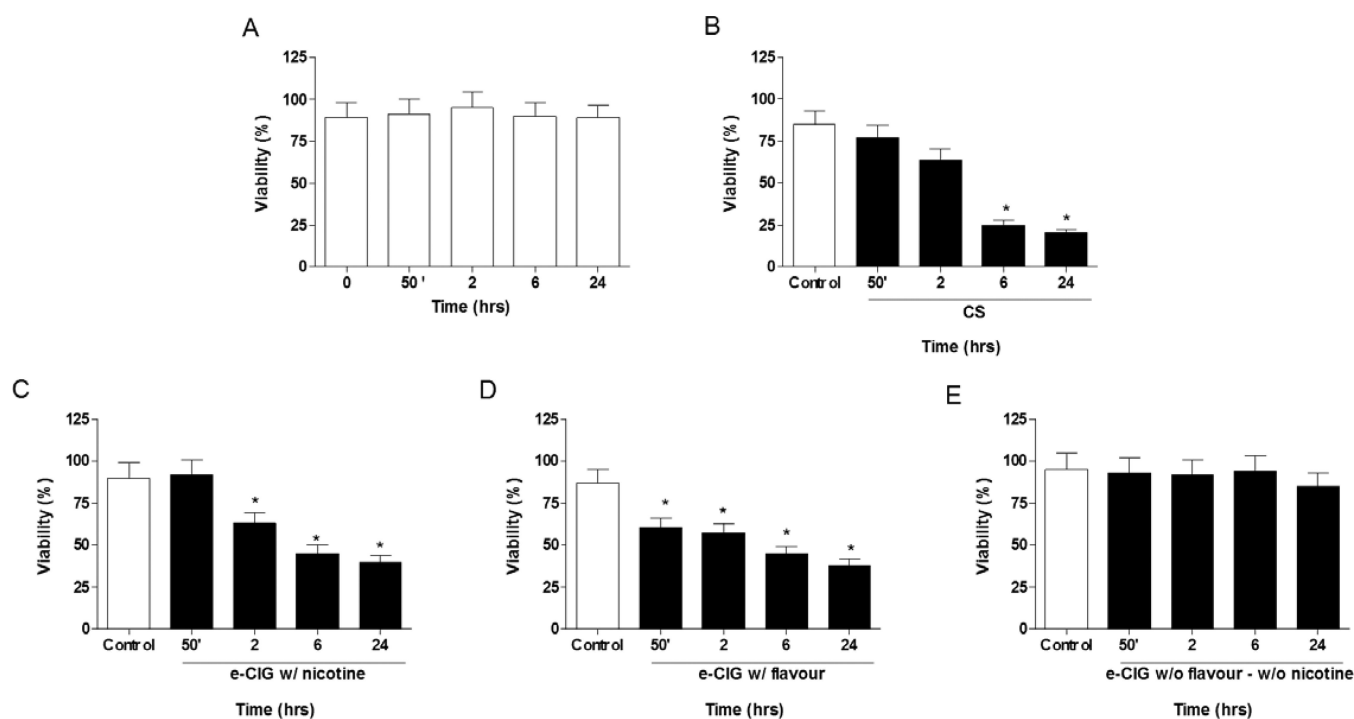
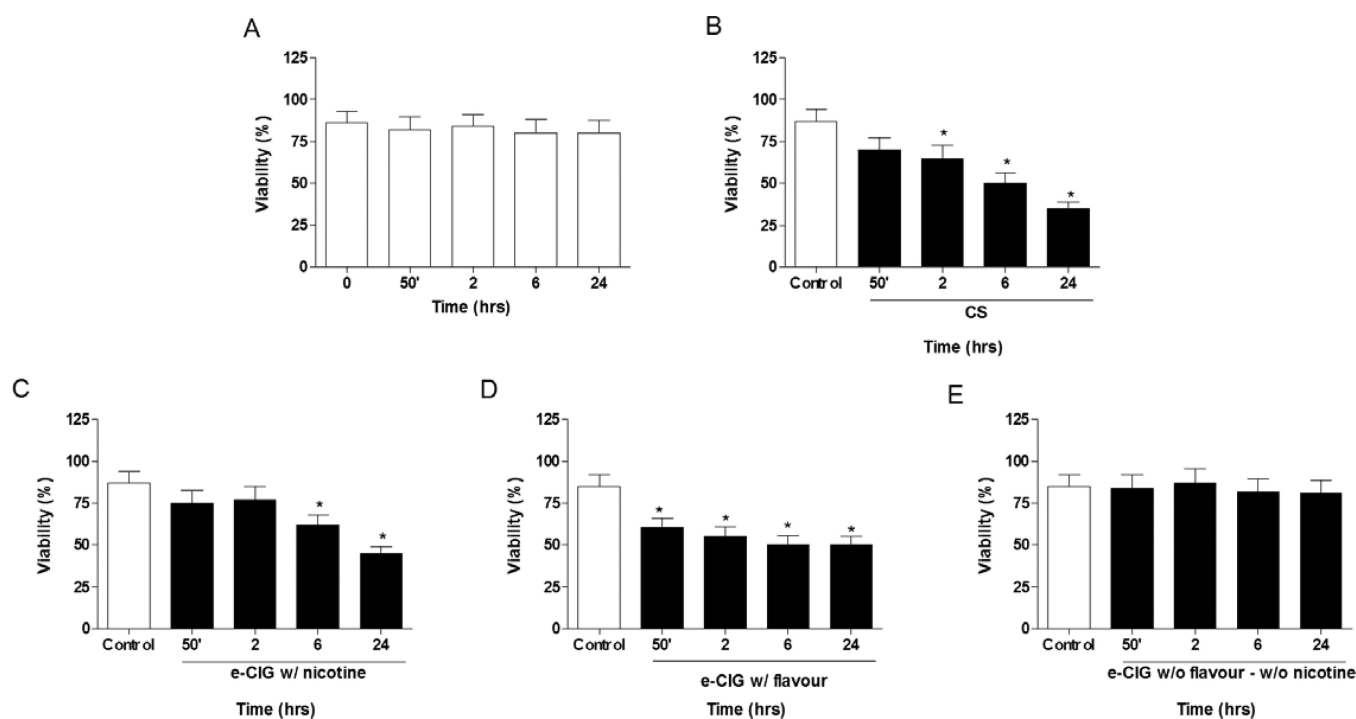


Figure 2.

Cytotoxicity measured as LDH release in A549 cells after exposure to air (A), cigarette smoke (B), electronic cigarette with nicotine (C), electronic cigarette with flavor (D) and only vaping (E). Triton X represent 100% of LDH release. Data are expressed as percentage of control (averages of five different experiments), *p < 0.05).

**Figure 3.**

Cell viability in HaCaT cells after exposure to air (A), cigarette smoke (B), electronic cigarette with nicotine (C), electronic cigarette with flavor (D) and only vaping (E). Data are expressed as percentage of control (averages of five different experiments), * $p < 0.05$).

**Figure 4.**

Cell viability in A549 cells after exposure to air (A), cigarette smoke (B), electronic cigarette with nicotine (C), electronic cigarette with flavor (D) and only vaping (E). Data are expressed as percentage of control (averages of five different experiments), * $p < 0.05$).

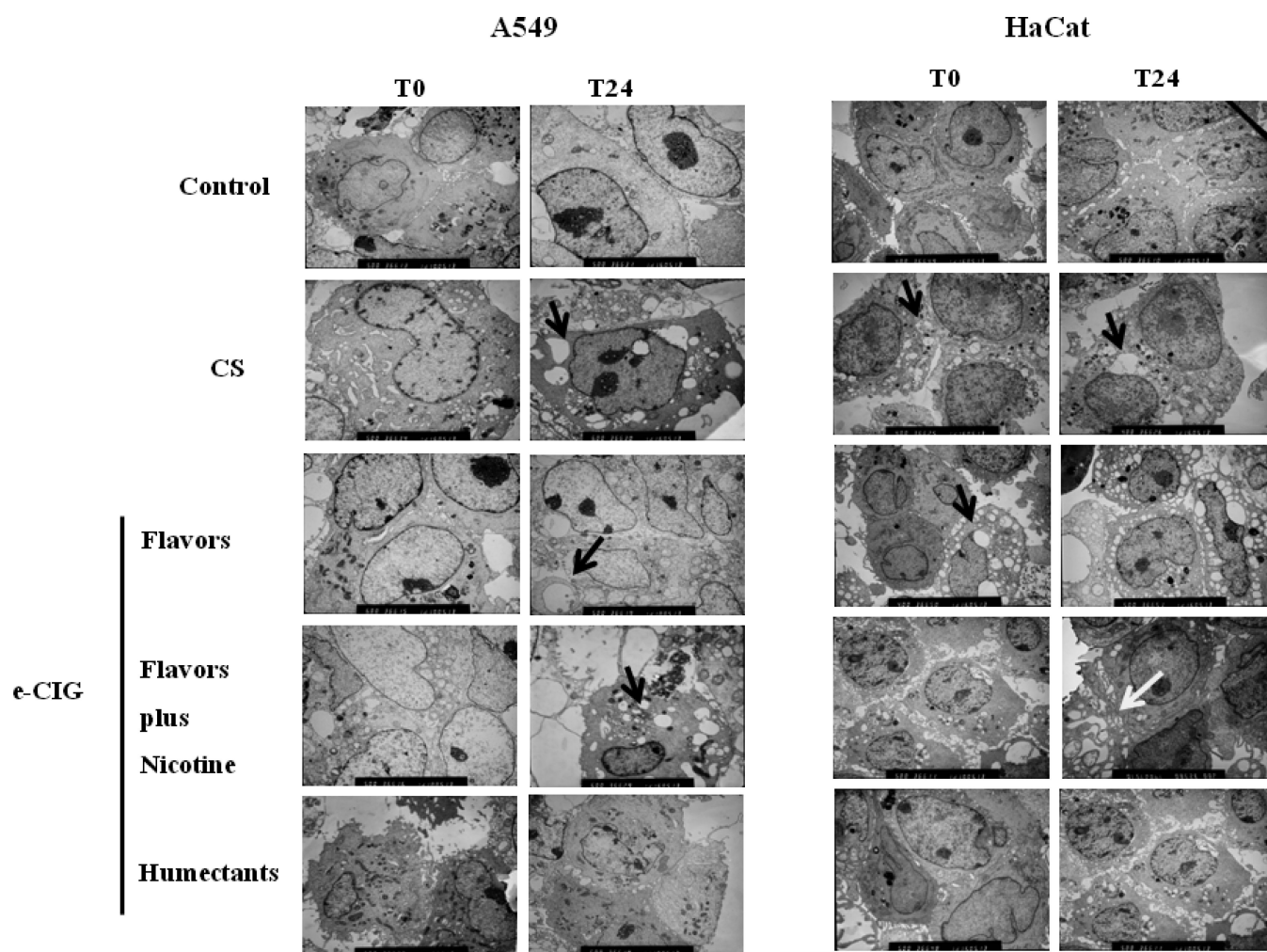


Figure 5. Ultrastructural study of A549 cells (left column) and HaCaT cells (right column) after exposure to different conditions at T0 (immediately after the exposure) and T24 (24 hr after the exposure). Bars = 2 μ m.

Table 1

Cytokine/chemokine concentrations in culture medium from untreated (control) and vapor exposed (e-cig) HaCat and A549 cells

	HaCat			A549		
	Control (pg/ml)	e-cig (pg/ml)		Control (pg/ml)	e-cig (pg/ml)	
Hu PDGF-BB	58.4	220.1	3.8	6.72	30.4	4.5
Hu basic FGF	31.6	64.7	2.0	24.4	240.8	9.9
Hu IL-1ra	49.7	157.7	3.18	41.1	32.7	0.8
Hu IL-6	1336.8	1002.6	0.8	6779.5	2704.12	0.4
Hu IL-8	504.5	6017.1	11.9	1135.8	1639.2	1.4
Hu IL-10	6.1	15.1	2.5	5.6	5.5	1.0
Hu IL-12	40.5	62.87	1.5	26.9	37.4	1.4
Hu IL-17	34.6	47.6	1.4	5.4	9.3	1.7
Hu G-CSF	12.9	125.7	9.7	11.5	12.6	1.1
Hu GM-CSF	23.3	50.6	2.2	7.4	12.9	1.7
Hu IFN- γ	57.1	188.5	3.3	61.3	50.3	0.8
Hu IP-10	245.3	3375.1	13.7	24.8	32.5	1.3
Hu MCP-1	470.4	1103.3	2.3	1437.3	2505.6	1.74
Hu MIP-1 β	20.6	41.8	2.0	5.7	10.1	1.8
RANTES	2487.2	2905.1	1.2	229.5	231.2	1.0
Hu TNF- α	10.2	35.1	3.4	12.3	8.8	0.7
Hu VEGF	3829.6	11689.1	3.1	3046.1	3488.1	1.1